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ORIGINAL ARTICLE

Reproductive Isolating Mechanisms in the Bangladesh Coastal Bullfrog *Hoplobatrachus litoralis* and Its Congeneric Species Revealed by Crossing Experiments and Examination on Spermatogenesis of the Hybrids

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Abstract To elucidate reproductive isolating mechanisms in the Bangladesh coastal bullfrog *Hoplobatrachus litoralis* and its congeneric species, we performed crossing experiments using three species: *H. litoralis*, *H. tigerinus* and *H. rugulosus*. In addition, we conducted histological observations on spermatogenesis of the hybrids. The reciprocal hybrids between *H. litoralis* and *H. tigerinus* developed normally with somewhat lower viability at the metamorphosis stage compared with the controls. Most of the metamorphosed frogs became mature. On the other hand, almost all hybrids between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus* died of underdevelopment at the tadpole stage, and only a few hybrids metamorphosed normally and survived to maturity. The inner structures of the testes of the control *H. litoralis* and *H. tigerinus* were completely normal, with seminiferous tubules filled with compact bundles of normal spermatozoa. Those of the reciprocal hybrids between *H. litoralis* and *H. tigerinus* were almost normal or slightly abnormal, with seminiferous tubules that contained pycnotic nuclei in addition to normal bundles of normal spermatozoa, which demonstrates slight abnormalities in spermatogenesis. In contrast, the hybrids between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus* had no bundles of spermatozoa nor spermatids in the seminiferous tubules, which indicates entirely abnormal spermatogenesis. Meiotic chromosome figures in the reciprocal hybrids between *H. litoralis* and *H. tigerinus* showed slight abnormalities, with the occurrence of univalents and increase of rod-shaped bivalents. These results indicate that *H. litoralis* and *H. tigerinus* are not isolated from each other by hybrid inviability nor by hybrid sterility, although the hybrids showed somewhat abnormal spermatogenesis in hybrids and that *H. rugulosus* is isolated from both *H. litoralis* and *H. tigerinus* by incomplete hybrid inviability and complete hybrid sterility.

Keywords *Hoplobatrachus*, reproductive isolation, interspecific hybrids, spermatogenesis, meiotic chromosomes

1. Introduction

Speciation, the process through which new species emerge, is one of the central topics of evolutionary biology, and is closely related to many biological disciplines, such as reproductive biology, genetics, and systematics. Therefore, persistent controversy regarding the definition of the term "species" may seem to be disconcerting (de Queiroz, 1998). Infact, accepted contemporary species concepts differ among researchers, and Mayden (1997, 1999) listed up to 24 named species concepts. Among them, the phylogenetic species concept and biological species concept are the most commonly advocated (Futuyma, 2005) and especially

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in herpetological systematics, the phylogenetic and evolutionary species concepts are frequently adopted (Matsui, 2011). However many researchers (Dobzhansky, 1950; Mayr, 1942; Wright, 1940) recommended the Biological Species Concept for multicellular organisms.

A species is a fundamental unit in biology (Mayr, 1982). The biological species concept emphasizes the role of reproductive isolation asserting that the integrity of sympatric species is maintained over time by reducing or directly impeding gene flow between individuals of different species. When populations are geographically isolated and there is no gene flow through interbreeding, the accumulation of intraspecies variation leads to speciation (allopatric speciation) (Hoskin *et al*., 2005; Mayr, 1963). In light of reproductive isolation being a fundamental aspect of the biological species, it is necessary to evaluate the degree of isolation among closely related species to elucidate the speciation process. Four nominal species have been described in the genus *Hoplobatrachus* from South and Southeast Asia (Frost, 2016): *H. crassus* in south to east India, Sri Lanka, Nepal and Bangladesh; *H. rugulosus* (= *H. chinensis*) in Myanmar, southern China, Taiwan, Thailand and peninsular Malaysia; *H. tigerinus* in east Afghanistan, Pakistan, India, Sri Lanka, Nepal, Bangladesh and Myanmar; and *H. litoralis* in the southeastern corner of Bangladesh. Bangladesh and the adjacent area of Myanmar (Teknaf) are only separated by the Naaf River, which probably does not provide a strong barrier preventing the migration of *Hoplobatrachus* species between Bangladesh and Myanmar.

Many cryptic species remain unstudied in Bangladesh due to homoplasy or phenetic similarities. Therefore, we revealed the occurrence of at least eight candidate frog species from Bangladesh, most or all of which might warrant full species status and two of them have been described (Hasan *et al*., 2012b, 2014). To further explore overlooked cryptic biodiversity in Bangladesh, we described *H. litoralis* as a new species from the southeastern corner of Bangladesh based on the morphological, ecological, and genetic data (Hasan *et al*., 2012a). This species is distinctly separated from its close relatives, *H. tigerinus* and *H. rugulosus*. However, its taxonomic status based on the biological species concept was not clarified. In this study, we clarified its relationships with *H. tigerinus* and *H. rugulosus* by crossing experiments and examination on spermatogenesis in the hybrid testes.

2. Materials and Methods

2.1 Specimens A total of 13 mature frogs that belong to the genus *Hoplobatrachus* were used in this study. The details of the specimens are shown in Table 1. We used the species names adopted by Frost (2016).

2.2 Artificial crossing experiments Artificial insemination was conducted for crossing experiments using the procedures described by Sumida *et al*. (2011) with a slight modification. Mature frogs were selected during the breeding season (June to July). Each mature female frog was induced to ovulate by injection into the body cavity with saline solution containing pituitary of *Lithobates catesbeianus* (commonly known as *Rana catesbeiana*) at a dose of one and a half pituitary gland per frog. The injected female frogs were monitored every 2 h interval to confirm the ovulation. The time from pituitary injection to accumulation of mature eggs in the oviducts was ca. 12 h in *H. litoralis* and *H. tigerinus* and ca. 13 h in *H. rugulosus*. The room temperature was ca. 26°C.

A sperm suspension was made by crushing testis removed from each male in a small volume of distilled water. After ovulation, eggs were squeezed out from the females, placed on glass slides, and fertilized with the sperm suspension. Then, the glass slides with fertilized eggs were submerged in Petri dishes that contained chlorine free water with an appropriate marking to identify the series for further handling. The hatched tadpoles were reared until around 3 weeks in Petri dishes in an incubator at 24°C and fed boiled spinach, and then moved to a cement tank in a greenhouse. They were

Table 1 List of frogs used for crossing experiments.

Species	Country	Location	No. of frogs			Abbreviation
			Female	Male	Total	
Hoplobatrachus litoralis	Bangladesh	Ukhia	1	4	5	Lit
Hoplobatrachus tigerinus	Bangladesh	Mymensingh	3	4	7	Tig
Hoplobatrachus rugulosus	Thailand	Chachoengaso	1	0	1	Rug

reared for about 2 months until metamorphosis. After metamorphosis, the froglets were transferred to facilitated terrarium (size: 40 × 60 × 15 cm^3) in the frog rooms at the Amphibian Research Center, Hiroshima University, and reared by feeding live crickets. At each stage, the normally developed embryos and tadpoles were counted, and the developmental capacity (survival rate) was calculated.

If any infection occurs, it is important to maintain water clean to limit the problem. Therefore, we checked the mortality and infection in each developmental stage so that necessary measures were taken to optimize the rearing technique for these frogs.

2.3 Observations of spermatogenesis The testes of mature males of the hybrids (ca. 3 yrs old) among three *Hoplobatrachus* species and the controls were used for histological observations of spermatogenesis. One testis was fixed with Navashin's solution, sectioned at 10 µm thickness, and stained with Heidenhain's iron hematoxylin; the other testis was used to make a chromosome preparations. Meiotic chromosomes were prepared according to the technique described by Schmid *et al*. (1979) with a slight modification. The chromosomes were stained with 4% Giemsa solution for 15 min. We observed the chromosomes at diakinesis and metaphase of the first reduction division, where bivalent and univalent chromosomes could be easily distinguished from each other. It is quite difficult to observe metaphase of the first reduction division of oocyte due to low cell density of ovary and very small volume ratio of nuclear region relative to the cytoplasmic region of oocyte, we only observed spermatogenesis to estimate possible post-mating isolation of the F1 hybrids.

3. Results

3.1 Developmental capacity The developmental capacity of the hybrids among three *Hoplobatrachus* species and the controls is shown in Table 2.

The hybrids between female *H. litoralis* and male *H. tigerinus* developed normally, but they had a lower rate of viability compared with the *H. litoralis* control at the metamorphosis stage (13.6% and 39.2%, respectively; Table 2, Figure 1A, B). The hybrids between female *H. tigerinus* and male *H. litoralis* also developed normally and had a slightly lower rate of viability compared with the *H. tigerinus* control at the metamorphosis stage (7.3% and 11.1%, respectively; Table 2, Figure 1C, D). After metamorphosis, they matured in 3 years (Figure 2A–D). In contrast, the hybrids between female *H. rugulosus* and male *H. litoralis* and the hybrids between female *H. rugulosus* and male *H. tigerinus* showed extremely low viability at the metamorphosis stage (0.6% and 0.2%, respectively; Table 2, Figure 1E, F). Almost all of these hybrids were underdeveloped, and only a few hybrids survived and matured (Figure 2E, F).

Both males and females were found in all hybrid series tested here and prominent differences of sex ratio among the controls and hybrid series were not observed.

3.2 Histological observation of testes The inner structures of the testes of the *H. litoralis* and *H. tigerinus* control males were completely normal, with seminiferous tubules filled with compact bundles of normal spermatozoa (Figure 3A, C). The testes of the reciprocal hybrids between *H. litoralis* and *H. tigerinus* were almost normal or slightly abnormal, with seminiferous tubules that contained pycnotic nuclei in addition to normal bundles of normal spermatozoa (Figure 3B, D), which demonstrates slight abnormalities in spermatogenesis. In contrast, the hybrids between female *H. rugulosus* and male *H. litoralis* and between female *H. rugulosus* and male *H. tigerinus* had no bundles of spermatozoa nor spermatids in the seminiferous tubules (Figure 3E, F), which demonstrates entirely abnormal spermatogenesis. This result indicates that, even if viable mature hybrids are produced, they are completely sterile.

3.3 Meiotic chromosomes observation Meiotic chromosomes were observed in mature male frogs of the reciprocal hybrids between *H. litoralis* and *H. tigerinus* and the controls. Chromosomes complements of the spermatocytes at the first stage of meiosis of the hybrids and the controls are shown in Figure 4. The diploid number of chromosomes in *Hoplobatrachus* is 26, and in the normal first meiotic division, chromosome complements consist of 13 bivalents, five large and eight small, which are either ring- or rod-shaped.

Meiotic spreads that differed in number of univalents are shown in Table 3. In the control *H. litoralis* and *H. tigerinus*, all (100%) of the 70 and 60 meiotic spreads, respectively, contained 13 bivalents and no univalents (Table 3, Figure 4A). In the hybrids between female *H. litoralis* and male *H. tigerinus*, of the 77 meiotic spreads analyzed, 74 (96.1%) contained 13 bivalents, two (2.6%) contained 12 bivalents and two univalents, and the remaining one (1.3%) contained 11 bivalents and four univalents (Table 3). The mean number of univalents per spermatocyte was 0.01 (Table 3). In the reciprocal hybrids between female *H. tigerinus* and male *H. litoralis*, of the 108 meiotic spreads analyzed, 101 (93.5%) contained

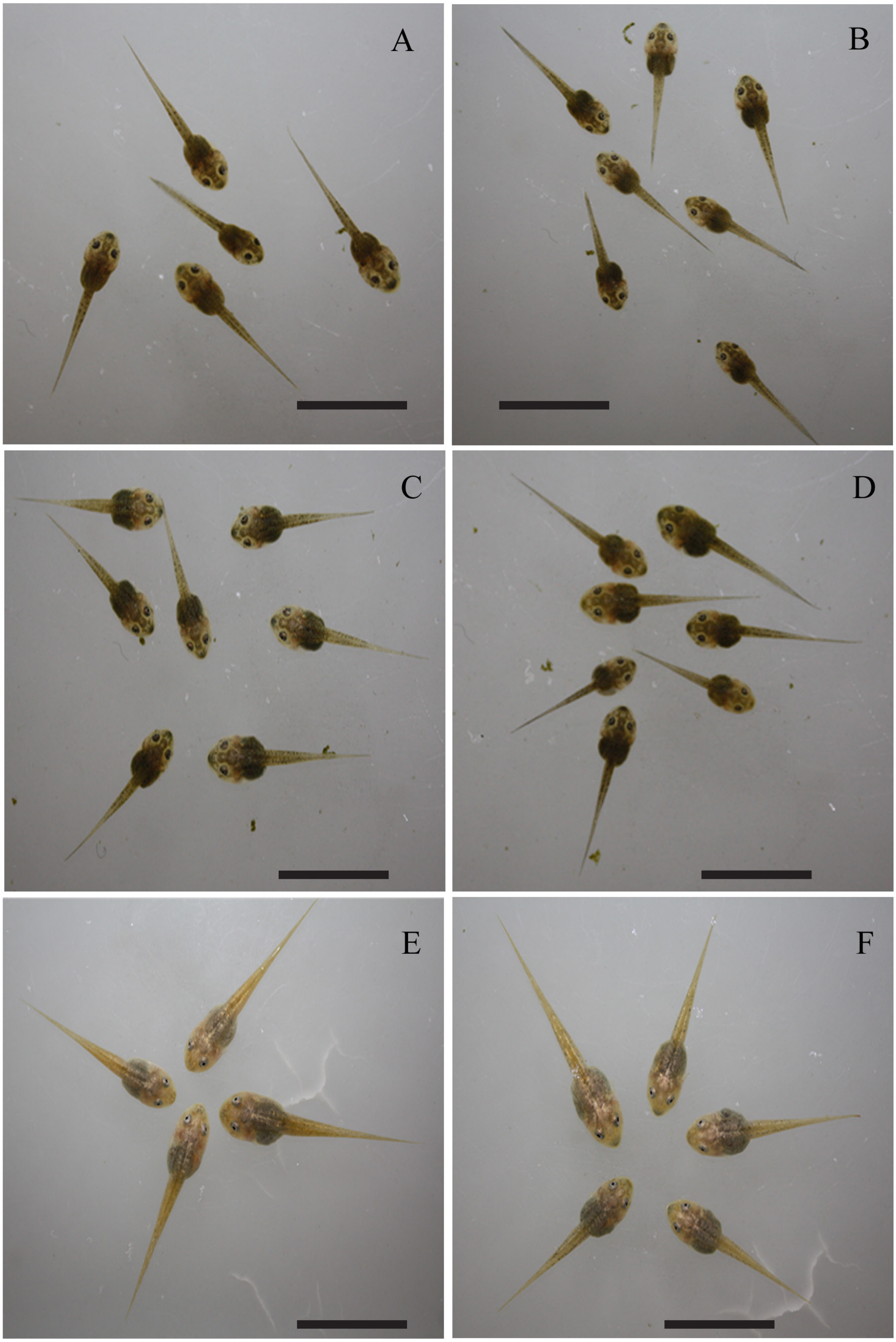

Figure 1 Tadpoles of interspecific hybrids among three *Hoplobatrachus* species and the controls. (A) Control *H. litoralis* (14 days old). (B) Hybrid between female *H. litoralis* and male *H. tigerinus* (14 day old). (C) Control *H. tigerinus* (14 days old). (D) Hybrid between female *H. tigerinus* and male *H. litoralis* (14 days old). (E) Hybrid between female *H. rugulosus* and male *H. litoralis* (38 days old). (F) Hybrid between female *H. rugulosus* and male *H. tigerinus* (38 days old). Scale bar = 10 mm.

Table 2 Developmental capacity of interspecific hybrids among three *Hoplobatrachus* species and the controls.

Date	Sex		Value						
	Females	Males	No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of 14 days old tadpoles (%)	No. of metamor-phosed frogs (%)
18 July, 2012	Lit1	Lit1A	220	209	208	203	183	159	102
	Lit1	Lit2	257	241	241	226	167	128	97
	Lit1	Lit3	270	268	267	263	217	196	95
	Lit1	Lit4	210	207	203	197	158	144	103
	Lit1	Lit1B	328	308	286	194	155	117	107
	Total		1285	1233 (95.9)	1205 (93.7)	1083 (84.3)	880 (68.5)	744 (57.9)	504 (39.2)
	Lit1	Tig1A	233	186	182	179	143	120	22
	Lit1	Tig2A	225	200	185	185	173	163	27
	Lit1	Tig3	303	288	235	231	179	171	12
	Lit1	Tig4	320	311	268	263	220	184	6
	Lit1	Tig1B	423	277	184	183	141	135	101
	Lit1	Tig2B	428	256	183	183	144	130	94
	Total		1932	1518 (78.6)	1237 (64.0)	1224 (63.4)	1000 (51.8)	903 (46.7)	262 (13.6)
	Tig1	Tig1A	367	342	277	267	91	52	45
	Tig1	Tig2A	366	352	226	211	147	93	60
	Tig1	Tig3	406	378	220	181	94	36	30
	Tig3	Tig1B	358	222	94	71	49	49	39
	Tig3	Tig2B	330	235	103	63	37	36	28
	Total		1827	1529 (83.7)	920 (50.4)	793 (43.4)	418 (22.9)	266 (14.6)	202 (11.1)
	Tig1	Lit1A	276	260	118	117	73	55	13
	Tig1	Lit2A	339	284	258	205	135	78	15
	Tig1	Lit3	358	329	256	242	177	87	12
	Tig2	Lit1	447	214	49	49	41	40	17
	Tig2	Lit2	446	260	0	0	0	0	0
	Tig3	Lit2B	358	250	164	127	74	50	40
	Tig3	Lit2B	408	313	204	187	116	106	94
	Total		2632	1910 (72.6)	1049 (39.9)	927 (35.2)	616 (23.4)	416 (15.8)	191 (7.3)
26 July, 2012	Rug1	Lit2 (1)	392	100	21	20	13	3	3
	Rug1	Lit2 (2)	377	106	29	25	18	2	2
	Rug1	Lit2 (3)	464	133	15	13	12	5	4
	Rug1	Lit2 (4)	303	87	14	10	7	1	0
	Total		1536	426 (27.7)	79 (5.1)	68 (4.4)	50 (3.3)	*11 (0.7)	9 (0.6)
	Rug1	Tig2(1)	376	89	48	40	21	5	1
	Rug1	Tig2(2)	431	107	41	34	20	4	1
	Rug1	Tig2(3)	362	98	40	36	16	1	1
	Rug1	Tig2(4)	360	82	46	42	21	0	0
	Total		1529	376 (24.6)	175 (11.4)	152 (9.9)	78 (5.1)	*10 (0.7)	3 (0.2)

* No. of tadpoles were count in 38 days instead of 14 days.

13 bivalents, five (4.6%) contained 12 bivalents and two univalents, and the remaining two (1.9%) contained 11 bivalents and four univalents (Table 3). The mean number of univalents per spermatocyte was 0.17 (Table 3).

Some meiotic spreads contained a few univalent and rod-shaped bivalents in addition to many ring-shaped bivalents (Figure 4B), whereas many rod- and ring-shaped bivalents were observed in Figure 4C. The numbers of ring- and rod-shaped bivalents are shown in Table 4. In the control *H. litoralis* and *H. tigerinus*, the frequencies of ring-shaped bivalents were 99.8% and 91.0%, respectively, with a mean number of 13 bivalents per cell; the frequencies of rod-shaped bivalents were 0.2% and 8.9%, respectively (Table 4). In the reciprocal hybrids between *H. litoralis* and *H. tigerinus*, the frequencies of ring-shaped bivalents were 76.9% and 74.5%, respectively, with mean numbers of 12.95 and 12.92 bivalents per cell, respectively; the frequencies of

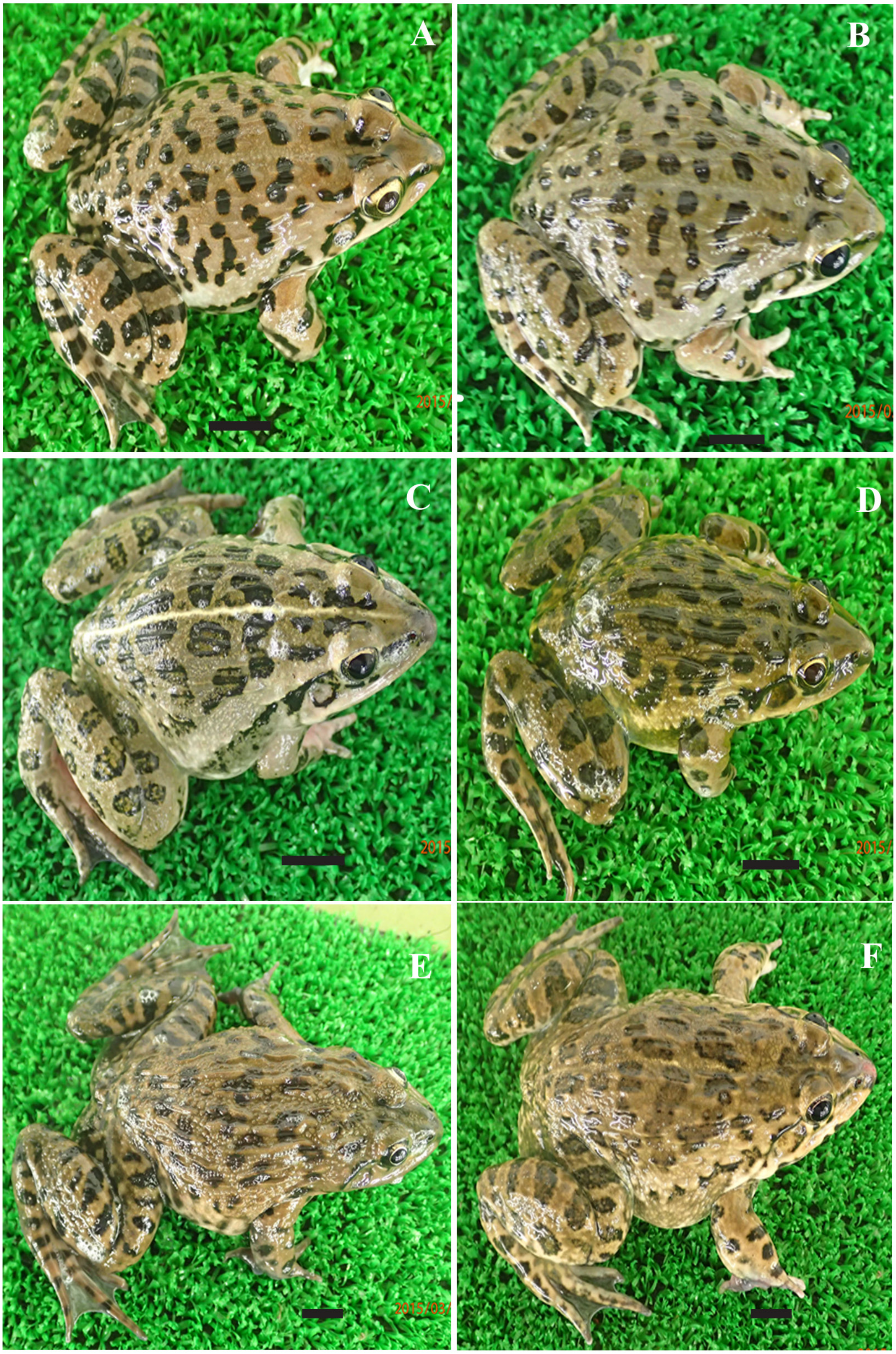

Figure 2 Dorsal views of 2 years and 8 months old frogs of interspecific hybrids among three *Hoplobatrachus* species and the controls. (A) Control *H. litoralis*. (B) Hybrid between female *H. litoralis* and male *H. tigerinus*. (C) Control *H. tigerinus*. (D) Hybrid between female *H. tigerinus* and male *H. litoralis*. (E) Hybrid between female *H. rugulosus* and male *H. litoralis*. (F) Hybrid between female *H. rugulosus* and male *H. tigerinus*. Scale bar = 10 mm.

Table 3 Number of meiotic spreads differing in number of univalents in male interspecific hybrids between two *Hoplobatrachus* species and the controls.

Sex		No. of meiosis	No. of univalents (%)					Mean no. of univalents per cell
Females	Males		0	2	4	6	8	
Lit	Lit	70	70 (100)					0
Lit	Tig	77	74 (96.1)	2 (2.6)	1 (1.3)			0.01
Tig	Tig	60	60 (100)					0
Tig	Lit	108	101 (93.5)	5 (4.6)	2 (1.9)			0.17

Table 4 Number of the ring- and rod-shaped bivalents in male interspecific hybrids between two *Hoplobatrachus* species and the controls.

Sex		No. of bivalents	Large chromosome		Small chromosome		Total		Mean No. of bivalents per cell
Female	Male		Ring (%)	Rod (%)	Ring (%)	Rod (%)	Ring (%)	Rod (%)	
Lit	Lit	910	348 (99.4)	2 (0.6)	560 (100)	0 (0)	908 (99.8)	2 (0.2)	13.0
Lit	Tig	997	349 (90.9)	35 (9.1)	418 (68.2)	195 (31.8)	767 (76.9)	230 (23.7)	12.9
Tig	Tig	780	270 (90.0)	30 (10.0)	440 (91.7)	40 (8.3)	710 (91.0)	70 (8.9)	13.0
Tig	Lit	1395	445 (82.9)	92 (17.1)	594 (69.2)	264 (30.8)	1039 (74.5)	356 (25.5)	12.9

rod-shaped bivalents were 23.7% and 25.5%, respectively (Table 4).

The controls of *H. litoralis* and *H. tigerinus* showed no reproductive isolation among their tadpoles. The reciprocal hybrids between *H. litoralis* and *H. tigerinus* developed normally with somewhat lower viability at the metamorphosis stage compared with the controls. Most of the metamorphosed frogs became mature. On the contrary, hybrids between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus* showed extremely low viability but grew up to adult stage (sterile).

4. Discussion

A simple definition of species is a group of inter-breeding natural populations that are reproductively isolated from other such groups (Mayr, 1969). Species are also viewed as genetic systems delimited by isolation mechanisms that prevent gene exchange (Dobzhansky, 1937; Mayr, 1942). These isolating mechanisms which serve to limit the gene flow between two species are categorized in terms of pre- or post-mating isolation mechanisms. If pre-mating isolation mechanisms fail, nature takes the help of second line of defence i.e. post-mating isolation mechanisms to bring about isolation among populations. Post-mating isolation (reproductive isolation) are likely to occur through a number of mechanisms like gametic incompatibility (sperm transfer takes place, but egg is not fertilized), zygotic mortality (egg is fertilized, but zygote does not develop), hybrid inviability (hybrid embryo forms, but of reduced viability), hybrid sterility (hybrid is viable, but resulting adult is sterile) and hybrid breakdown (first generation [F1] hybrids are viable and fertile, but further hybrid generations [F2 and back crosses] may be inviable or sterile) (Mallet, 1998; Mayr, 1970). Reproductive isolation is necessary for morphological, ecological, and genetic divergence between both sympatric and allopatric groups (Turelli *et al*., 2001). Genetic divergence results in reproductive isolation that reflects further accumulation of genetic differentiation at many loci over a long period of time (Ferguson, 2002; Sites and Marshal, 2003, 2004). Generally, it is believed that large numbers of genes are responsible for reproductive isolation between taxa, and gradual changes occur at all of these loci over time. As a result, genetic divergence is correlated with degree of reproductive isolation (Wu and Holloncher, 1998). These kinds of correlations have been demonstrated in *Drosophila* (Coyne and Orr, 1989), the salamander *Desmognathus ochrophaeus* (Tilley *et al*., 1990), and

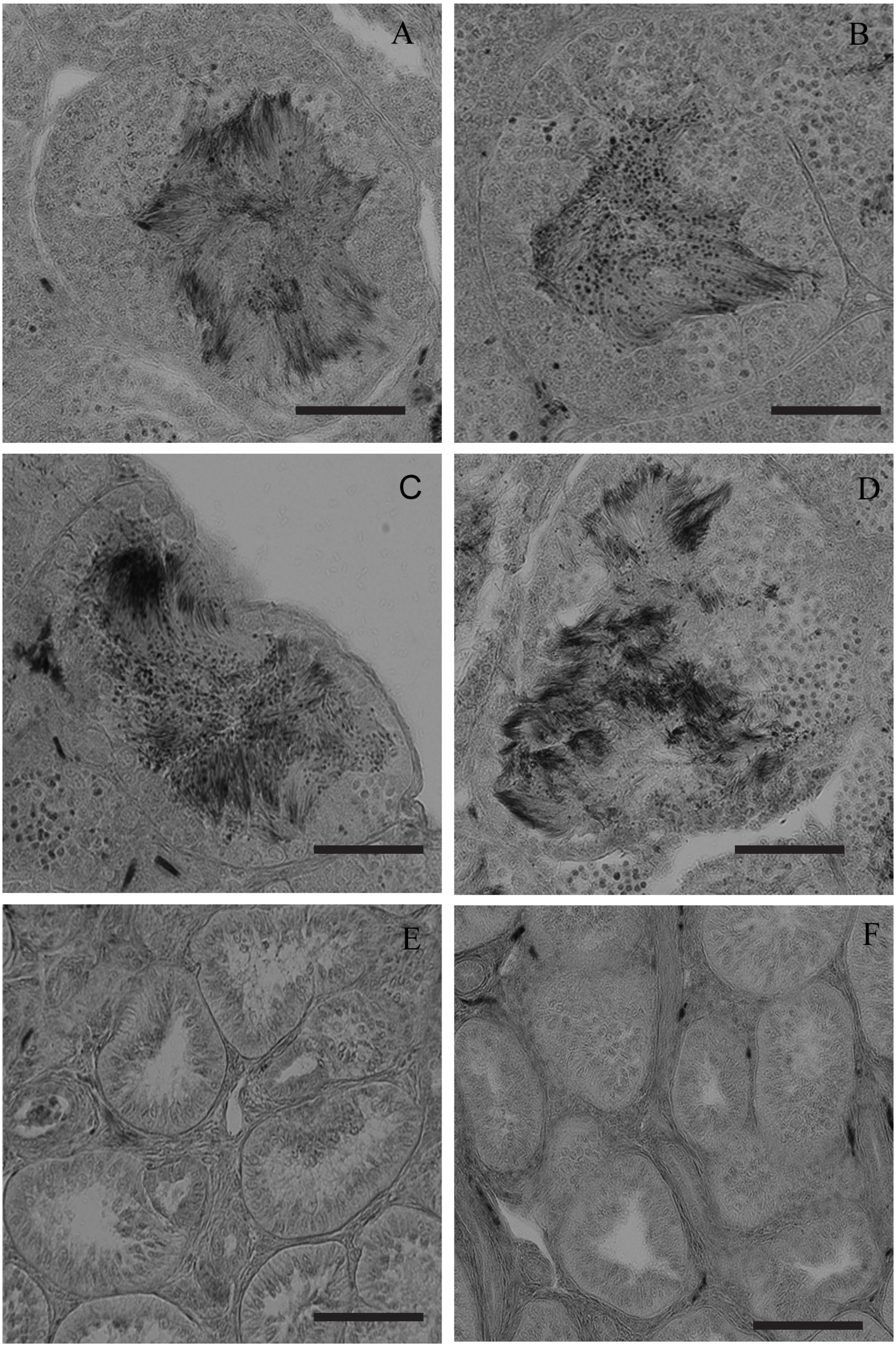

Figure 3 Cross sections of seminiferous tubules in the testes of interspecific hybrids among three *Hoplobatrachus* species and the controls. (A) Control *H. litoralis.* (B) Hybrid between female *H. litoralis* and male *H. tigerinus.* (C) Control *H. tigerinus*. (D) Hybrid between female *H. tigerinus* and male *H. litoralis*. (E) Hybrid between female *H. rugulosus* and male *H. litoralis*. (F) Hybrid between female *H. rugulosus* and male *H. tigerinus*. Scale bar = 15 μm.

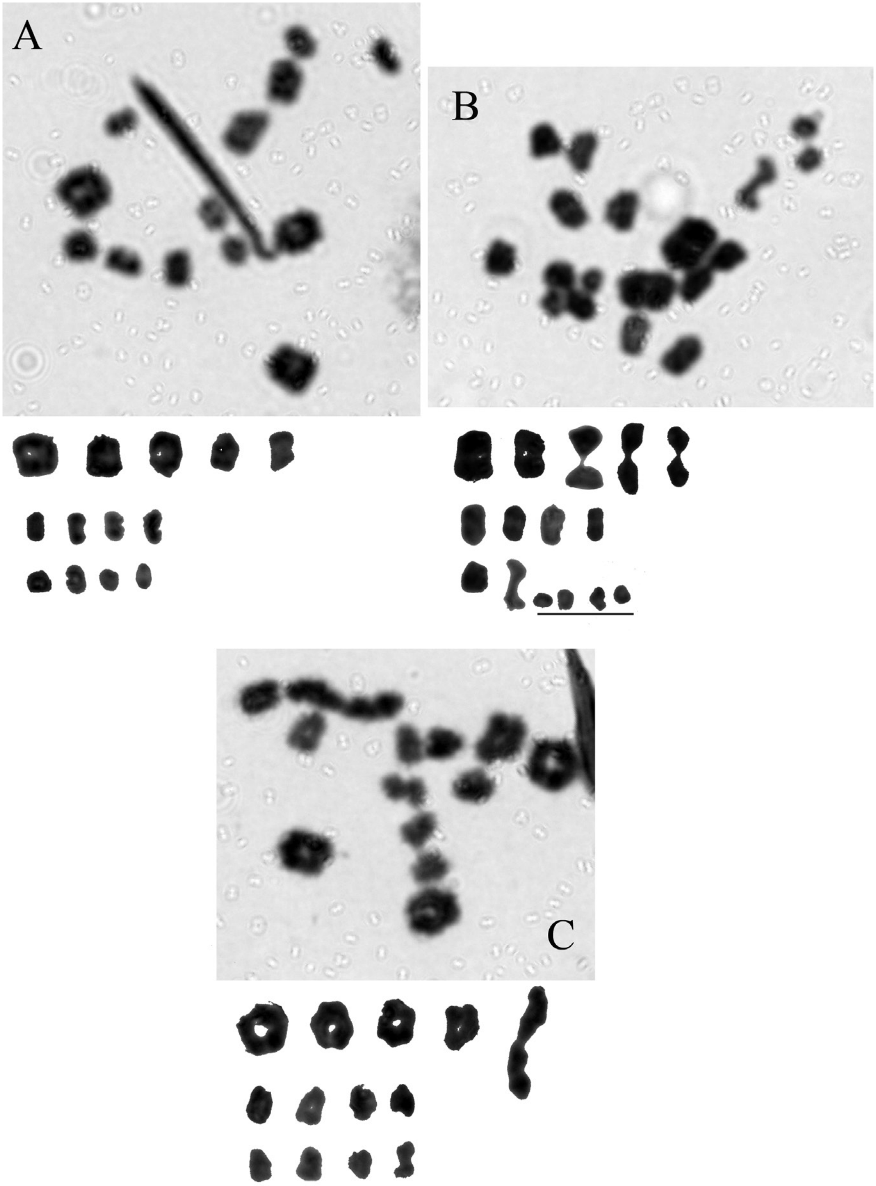

Figure 4 Spermatocytes at the first meiosis and chromosome complements in interspecific hybrids between two *Hoplobatrachus* species and the control. Bars under the chromosomes indicate univalent. (A) Control *H. litoralis* containing 13 bivalents, which are all ring-shaped. (B) Hybrid between female *H. litoralis* and male *H. tigerinus* containing 11 bivalents, which are ring- and rod-shaped and 4 univalents. (C) Hybrid between female *H. tigerinus* and male *H. litoralis* containing 13 bivalents, which are ring- and rod-shaped.

some anuran species (Sasa *et al*., 1998).

In the present study, crossing experiments demonstrated that reciprocal hybrids between *H. litoralis* and *H. tigerinus* were viable at the tadpole stage and underwent normal metamorphosis. Most of the metamorphosed frogs grew up and matured when maintained in the laboratory for 3 years. Almost all hybrids between female *H. rugulosus* and male *H. tigerinus* or *H. litoralis* died of underdevelopment at the tadpole stage, and only very few hybrids metamorphosed normally and survived to maturity. These results indicate that *H. litoralis* and *H. tigerinus* are not isolated from each other by hybrid inviability, but *H. rugulosus* is isolated from both *H. litoralis* and *H. tigerinus* by incomplete hybrid inviability.

Hoplobatrachus litoralis was first described from the southeastern corner of Cox's Bazar district in Bangladesh (Hasan *et al*., 2012a) and is a large frog, with SVL of 81.3–102.1 mm in males and 83.2–121.3 mm in females. This species has distinct morphological differentiation from its near congeners *H. tigerinus* and *H. rugulosus*. Based on the mitochondrial DNA sequence data, *H. litoralis* was shown to be 3.2% and 14.2% (for 16S rRNA and Cytb genes, respectively) genetically divergent from *H. tigerinus* and was 5.0% and 16.3% divergent from *H. rugulosus*. Reproductive isolation based on hybrid inviability also reflects the degree of genetic divergence; *H. litoralis* produced viable reciprocal hybrids with *H. tigerinus* at metamorphosis rates of 13.6% and 7.3%, which were lower than that of the controls. However, *H. litoralis* produced many inviable hybrids with *H. rugulosus*; there were very few survivors and a metamorphosis rate was only 0.6%, which indicates high degree of reproductive isolation from *H. rugulosus.*

Regarding the histological observation, we found that seminiferous tubules of the *H. litoralis* and *H. tigerinus* controls were filled with a lot of compact bundles of normal spermatozoa. In the reciprocal hybrids between *H. litoralis* and *H. tigerinus*, there were normal bundles of spermatozoa, but the density was lower. In addition, pycnotic nuclei were observed in these hybrids, which indicate some abnormalities in the process of meiosis. No spermatozoa were observed in the hybrids between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus*, which indicates that these hybrids were sterile males, which is probably caused by preventing normal chromosome pairing and segregation during meiosis.

In the meiotic chromosome observation, normal states with 13 bivalents were found in both *H. litoralis* and *H. tigerinus* controls, with more than 90% ring-shaped bivalents. However, spermatogenesis in the reciprocal hybrids between *H. litoralis* and *H. tigerinus* showed slight abnormalities as shown by the occurrence of univalents and an increased ratio of rod-shaped bivalents in both large and small chromosomes. These results show that *H. litoralis* and *H. tigerinus* are not isolated from each other by hybrid inviability nor by hybrid sterility, but show a slight divergence indicated by somewhat abnormal spermatogenesis. *Hoplobatrachus rugulosus* is isolated from both *H. litoralis* and *H. tigerinus* by incomplete hybrid inviability and complete hybrid sterility. This should be confirmed by future studies using males of *H. rugulosus*.

According to Sumida *et al*. (2003), two different species are separated from each other either completely or incompletely by gametic isolation: if gametic isolation is incomplete, the two species are completely or incompletely isolated by hybrid inviability; if hybrid inviability is incomplete, the two species are completely isolated by hybrid sterility. If we follow the criteria of Sumida *et al*. (2003), it is reasonably argued that *H. litoralis* is s distinct species from *H. rugulosus*. Considering the present results as well as the recent inter- and intra-specific genetic divergence in the *Hoplobatarchus* species group using microsatellite marker (Sultana *et al*., 2016) together with the morphological and genetic variations reported by Hasan *et al*. (2012a), it seems reasonable to regard *H. litoralis* as a distinct species. Similar degree of reproductive isolation also obtained from the two distinct species of Ryukyu Islands, Japan: *Babina holsti* and *B. subaspera* (Kakehashi *et al*., unpublished).

The most intriguing finding in the present study is the production of interspecific sterile hybrids between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus*. Our results are consistent with those of Sasa *et al*. (1998) and Alam *et al*. (2012), who found that hybrid sterility in anurans appears to evolve more rapidly than hybrid inviability. Because the spontaneous production of allotriploids was discussed in our previous study (Alam *et al*., 2012), we did not focus on this issue in the present study. Further, Sumida *et al*. (2007) reported that the phylogenetic relationships among taxa are closely related to the degree of reproductive isolation, and the present results also indicated that close relationships observed in phylogenetic trees (Hasan *et al*., 2012a) are reflected by the crossing experiments.

The present study addressed the strength of reproductive isolation among three *Hoplobatrachus* species and confirmed that some degree of reproductive isolation exists between *H. litoralis* and *H. tigerinus*, and complete isolation exists between female *H. rugulosus* and male *H. litoralis* or *H. tigerinus*. Finally, and most importantly, *H. litoralis* seems to be endemic in the coastal belt (Teknaf Peninsula) of Bangladesh and no attempt has been previously made to produce offspring in laboratories by artificial breeding techniques. Recently, the ecosystem of this peninsula is adversely changing due to anthropological activities and a few marine turtles (e.g., *Chelonia mydas*, *Eretmochelys imbricata* and so on) are listed as globally threatened by IUCN from this area. Therefore, it is necessary to take measure to conserve this frog in nature. In this study, we optimized the breeding technique for this species and are able to breed this frog in captivity. This information should be useful for further conservation planning for this endemic species. In addition, the Wildlife Management and Nature Conservation Division, local government, and non-government organizations in Bangladesh should take immediate action to preserve the natural breeding ground and ecology of this species.

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